Proteomics and antioxidant enzymes reveal different mechanisms of toxicity induced by ionic and nanoparticulate silver in bacteria†

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The increased use of silver nanoparticles (AgNPs) raises concerns about their impacts on aquatic ecosystems. The impacts of Ag+ and AgNPs were assessed on proteomic and antioxidant enzymatic responses of Pseudomonas sp. M1. The effects of Ag+ on bacterial growth were stronger than those of AgNPs (EC20 = 107.1 μgL−1 for Ag+; EC20 = 307.2 μgL−1 for AgNPs), indicating the lower toxicity of the latter. At EC20, the activities of antioxidant enzymes increased more under exposure to Ag+ than to AgNPs, particularly for superoxide dismutase and glutathione peroxidase (stimulation of 667% and 433%, respectively). A total of 166 proteins were identified by SWATH-MS; among these, only 59 had their content significantly altered by one or both forms of silver. Exposure to AgNPs resulted in an increase of about 54% of these proteins, whereas 54% decreased under exposure to Ag+. Gene Ontology enrichment analysis revealed that protein folding and transmembrane transport were the most relevant processes affected by Ag+ exposure, whereas AgNPs mostly affected translation. Also, results suggest that each form of silver induced different adaptive responses. Furthermore, the low levels of Ag+ released from AgNPs (<0.1%) support a minor role of dissolved silver in AgNP toxicity to Pseudomonas sp. M1.

Environmental significance
As the use of AgNPs increases, aquatic ecosystems can be a repository of this widely disseminated antimicrobial agent. Aquatic bacteria that play a crucial role in organic matter decomposition may come in contact with AgNPs through contaminated waters or contaminated organic matter. Moreover, it is still unclear whether the toxicity of AgNPs is related to their nanoparticulate form and/or mediated by Ag+ released from AgNP dissolution. Based on proteomics, our study reveals that each form of Ag induced different adaptive responses in the metabolic, energetic and stress pathways. Of particular significance is the increased activity of superoxide dismutase and glutathione peroxidase, supporting the role of these enzymes in facing the oxidative stress induced by both forms of Ag.

Introduction
The developments in nanotechnology over the past decade increased concerns about the environmental impacts of engineered nanoparticles (ENPs). Silver nanoparticles (AgNPs) are among the most widely used ENPs.1 Due to their broad-spectrum antimicrobial properties, AgNPs have been used extensively in textiles and therapeutics.2,3 About 20–130 tons of ionic silver (Ag+) were predicted to reach EU freshwaters per year, mainly due to ionic leaching of AgNPs from biocidal plastics and textiles.4 The industrial production of AgNPs was predicted to become ca. 20 tons per year,5 of which about 30% can be released to the aquatic environment.6 The environmental concentrations of AgNPs in freshwaters are predicted to be lower than 1 μgL−1 but may increase up to 100 times in wastewater effluents.7,8 Furthermore, the possibility of attaining higher AgNP concentrations due to accidental spills cannot be ignored. Hence, AgNPs have been considered emerging chemical contaminants in aquatic environments and recommended for environmental risk assessment.9

The prime challenge regarding the assessment of ENP toxicity in aquatic biota is still the establishment of standard nano-specific protocols,10 including sample preparation and test conditions, to achieve reproducible results and to effectively link the toxicological information to the physicochemical properties of ENPs (but see test guidelines11).
Many studies have reported the toxic effects of AgNPs on freshwater organisms, including bacteria, algae, fungi, invertebrates and fish, but little is known about the mechanisms underlying such effects. Some studies indicate that the bioavailable Ag⁺ released from AgNPs contributes to toxicity. Speciation of released Ag⁺ in aquatic environments might be a key factor influencing toxicity and may occur via interactions with natural ligands, forming AgCl, Ag₂O, Ag₂S, and other silver complexes. Lubick showed that some algal species are more sensitive to AgNPs than to free Ag⁺, but when cysteine (a metal chelator) is added, the toxicity of both silver forms is reduced due to complexation of Ag⁺. More recently, it was shown that actual Gibbs free energy can be used to accurately calculate the equilibrium concentrations of AgNPs and released Ag⁺ and precisely determine the real transformation, fate, and ecotoxicity of AgNPs in aquatic environments.

Heterotrophic bacteria play a crucial role in stream ecosystem processes, and they can be affected by acute and chronic exposure to AgNPs. Very small particle size AgNPs can exhibit higher toxicity to prokaryotic cells than Ag⁺ or other forms of Ag. Chronic exposure to Ag⁺ released from AgNPs at very low concentrations may also heighten the silver tolerance/resistance in bacteria. Interactions with and adhesion of AgNPs to the bacterial surface can alter cell wall structure, and induce intracellular generation of reactive oxygen species (ROS), disruption of the plasma membrane, changes in protein interactions, and interference with DNA replication, and damage macromolecules (lipids, proteins, DNA and RNA).

Environmental stress biomarkers and omics are among the most promising next-generation toxicity assessment tools, enhancing measurements of direct and highly sensitive responses to emerging environmental contaminants at the cellular and sub-cellular levels. Freshwater microbes and invertebrates are reported to trigger antioxidant defence mechanisms in response to exposure to metals or metal-based ENPs; these include changes in the activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-S-transferase (GST), suggesting a role as oxidative stress biomarkers. These enzymes are closely associated with the ascorbate-glutathione cycle, in which the reduced form of glutathione (GSH) is converted to its oxidized form (GSSG), thus maintaining a high GSH:GSSG ratio.

To gain insights into the mechanisms underlying the impacts of Ag⁺ and AgNPs on Pseudomonas sp. M1 and its ability to deal with these toxicants, we determined i) dose-response curves using bacterial growth as endpoint, ii) antioxidant enzymatic responses, and iii) proteomic responses. We hypothesized that i) Pseudomonas sp. M1 would exhibit more tolerance to AgNPs than to Ag⁺, ii) the response profiles of antioxidant enzymes would potentially indicate the oxidative stress induced by the two silver forms, and iii) proteomic profiles would portray the key signature proteins in response to Ag⁺ and AgNPs. Moreover, we aim to elucidate the role of dissolved ionic silver released from AgNPs in the overall toxicity effects as well as their underlying mechanisms.

**Materials and methods**

**Bacterial exposure to Ag⁺ and AgNPs**

*Pseudomonas* sp. M1 is an oxidase-positive strain isolated from sediments of the Rhine River (Wageningen, Netherlands) and phylogenetically close to the environmental strain *P. citronellolis*. *Pseudomonas* sp. M1 cells were cultivated at 30 °C and maintained in *Pseudomonas* isolation agar (PIA) plates. For the exposure experiments, liquid cultures were prepared in sterile 250 mL Erlenmeyer flasks (pre-silanized to avoid adherence of metals/ENPs to the surface) with 50 mL of mineral medium (MM) as described by Hartmans et al. and supplemented with 0.4% lactate as sole carbon source under orbital shaking (200 rpm; Certomat BS 3, B. Braun Biotech International) at 30 °C. The inoculum consisted of bacterial cells grown to an optical density (OD₆₀₀) of between 0.6 and 0.8 to reach the exponential growth phase and diluted in fresh MM containing lactate to obtain an initial OD₆₀₀ of 0.15.

An aqueous suspension of citrate-coated AgNPs (1 g L⁻¹) was purchased from NanoSys GmbH (Wolfhalden, Switzerland). AgNO₃ (>99%) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified. A stock suspension of AgNPs (100 mg L⁻¹) was prepared in filtered (0.2 μm pore-size membrane; Millipore, Billerica, MA) ultrapure water (Milli-Q, 18.2 MΩ cm) and stored in the dark. A stock solution of AgNO₃ was prepared by suspending the powder in autoclaved (120 °C, 20 min) ultrapure water.

The estimations of the effective concentrations (EC₁₀ and EC₂₀), using growth as endpoint, were performed by exposing *Pseudomonas* sp. M1 cells to increasing concentrations of AgNPs (≤1000 μg L⁻¹) and to their ionic precursor (Ag⁺ in AgNO₃; ≤300 μg L⁻¹) for 90 min (at 30 °C, 200 rpm). For enzymatic and proteomic assays, cells at mid-exponential...
phase\textsuperscript{32-38} were exposed to toxicants (under same conditions). For enzymatic assays, cells were incubated with or without i) Ag\textsuperscript{+} at concentrations similar to EC\textsubscript{10} and EC\textsubscript{20} and ii) AgNPs at concentrations similar to EC\textsubscript{10} and EC\textsubscript{20} for AgNPs and the concentrations used for exposure to Ag\textsuperscript{+}. For proteomic analysis, the cells were incubated under similar conditions with or without Ag\textsuperscript{+} or AgNPs at the concentrations of their respective EC\textsubscript{20}.

A complementary test was carried out with cysteine (1-cysteine, \(\geq 98\%\), Sigma-Aldrich), a strong Ag\textsuperscript{+} chelating agent, to better understand the actual role of released Ag\textsuperscript{+} in AgNP-induced toxicity. The effects of 500 \(\mu\)M cysteine\textsuperscript{14} on the growth of \textit{Pseudomonas} sp. M1 in mineral medium with 0.4\% lactate (30 °C for 90 min) was assessed in the presence or absence of Ag\textsuperscript{+} (at EC\textsubscript{20}) or AgNPs (at EC\textsubscript{20}).

Characterization of nanoparticles and quantification of dissolved silver

The stock suspension of AgNPs was characterized using a UV-vis spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan). The hydrodynamic size distribution, dispersity and stability of nanoparticles in the aqueous stock suspension before the experiment and in the exposure medium at the beginning and end of the experiment were monitored by dynamic light scattering (DLS) and zeta potential using a Zetasizer (Malvern, Zetasizer Nano ZS, Malvern Instruments Limited, UK).

Total and dissolved Ag\textsuperscript{+} derived from AgNPs in the growth medium containing AgNPs were quantified at the beginning and at the end of the experiment by inductively coupled plasma mass spectrometry (Thermo X7 Q-ICP-MS, Thermo Scientific) at the Scientific and Technological Research Assistance Centre (C.A.C.T.I., University of Vigo, Spain).

Bacterial cells were removed from the medium by centrifugation (5000g, 5 min). Total Ag concentration (isotope\textsuperscript{109}Ag) in the medium was determined in cell-free supernatant diluted with HNO\textsubscript{3} (2\%). The quantification of dissolved Ag\textsuperscript{+} from AgNPs was performed in cell-free supernatant after ultrafiltration for 30 min (3220g, 3 times) using Amicon Ultra-15 centrifugal filter units (Merck Millipore, Germany; 3 kDa molecular weight cut-off corresponding to <2 nm of estimated pore size) followed by acidification with HNO\textsubscript{3} (1.68\% final concentration).

Preparation of cell-free extracts and protein quantification

Cells of \textit{Pseudomonas} sp. M1 were harvested by centrifugation (15 000g for 10 min at 4 °C). For the assessment of the activities of antioxidant enzymes, cells were washed twice with ice-cold washing buffer (10 mM Tris-HCl, pH 7.0, 0.25 M sucrose) and resuspended into the sonication buffer (10 mM Tris-HCl, pH 7.0). For the proteomic analysis, 10 mM Tris-HCl (pH 7.4) with 0.25 M sucrose was used as washing buffer, whereas sucrose was not present in the sonication buffer. Cells were disrupted by ultrasonication at 20 kHz (15 min, cycles of 3 s burst with a 9 s interval, in ice) using a 13 mm probe and an Ultrasonic Processor GEX 400 (Sonics and Materials Inc, CT, USA). Unbroken cells were removed by centrifugation (5000g for 15 min at 4 °C), and cell-free extracts were obtained by centrifugation at 16 000g for 30 min at 4 °C followed by filtration (0.2 \(\mu\)m; Millipore, Billerica, MA). The extract was stored at −80 °C until use.

Protein concentration in the cell-free extracts was determined by a modified Lowry method\textsuperscript{15} using bovine serum albumin (BSA) as protein standard.

Antioxidant enzyme activities

Antioxidant enzyme activities from unexposed cells were compared with those from cells exposed to Ag\textsuperscript{+} or AgNPs by spectrophotometry (SpectraMax Plus 384 Microplate Reader, Molecular Devices, CA, USA). The activity of SOD was assessed based on its ability to inhibit superoxide radical dependent reactions using the Ransod Kit (Randox Laboratories Limited, Crumlin, UK). Briefly, 10 \(\mu\)L of the cell-free extract was added to 165 \(\mu\)L of reaction mixture containing 0.05 mM xanthine, 0.025 mM 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) dissolved in 40 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 10.2) and 0.94 mM ethylenediaminetetraacetic acid (EDTA). The formation of superoxide radicals from xanthine started after the addition of 25 \(\mu\)L of xanthine oxidase (80 U L\textsuperscript{−1}).

The activity of CAT was determined as described by Clairborne.\textsuperscript{43} 25 \(\mu\)L cell-free extract was added to a 275 \(\mu\)L reaction mixture comprising 0.05 M potassium phosphate buffer (pH 7.0) and 30 mM H\textsubscript{2}O\textsubscript{2}. The decrease in absorbance due to dismutation of H\textsubscript{2}O\textsubscript{2} was detected at 240 nm (\(\epsilon = 0.04\) mM\textsuperscript{−1} cm\textsuperscript{−1}). CAT activity was calculated from the slope of the H\textsubscript{2}O\textsubscript{2} absorbance curve and normalized to the protein concentration.

The GPx activity was determined according to Flohé and Günzler\textsuperscript{44} with slight modifications.\textsuperscript{28} Cell-free extract (10 \(\mu\)L) was added to the reaction mixture (290 \(\mu\)L) containing 0.05 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}, 1 mM GSH (reduced glutathione), 0.24 mM reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), 0.25 mM H\textsubscript{2}O\textsubscript{2} and 0.2 U glutathione reductase (GR, from yeast). H\textsubscript{2}O\textsubscript{2} served as the substrate while Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} blocked the activity of catalase. The oxidation of NADPH was detected at 340 nm (\(\epsilon = 6.2\) mM\textsuperscript{−1} cm\textsuperscript{−1}) when GR reduced GSSG (oxidized glutathione) to GSH. The activity of GPx was calculated from the slope of the NADPH absorbance curve and normalized to the protein concentration.

The activity of GST in \textit{Pseudomonas} sp. M1 was determined according to Habig \textit{et al}.\textsuperscript{45} by measuring the formation of 1-glutathion-2,4-dinitrobenzene resulting from the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB). The cell-free extract (50 \(\mu\)L) was added to 250 \(\mu\)L of reaction mixture containing 0.1 M potassium phosphate buffer (pH 6.5), 1.5 mM CDNB and 1.5 mM GSH. The activity of GST was calculated from the slope of the absorbance curve at 340 nm (\(\epsilon = 9.6\) mM\textsuperscript{−1} cm\textsuperscript{−1}) and normalized to the protein concentration.
Protein denaturation, SDS-PAGE and gel staining

Prior to protein identification, volumes equivalent to 200 μg of total protein from each sample were incubated for 60 min at room temperature with urea loading buffer containing 9 M urea, 50 mM Tris at pH 8.8, 10% (v/v) glycerol, 10% (w/v) SDS, 0.002% (w/v) bromophenol blue and 20 mM dithiothreitol (DTT). A second incubation was done for 60 min at room temperature with the same buffer containing 20 mM acrylamide. Then, samples were loaded on SDS-polyacrylamide gels and subjected to electrophoretic separation. Finally, gels were stained with Coomassie Brilliant Blue G-250 solution. Detected protein bands were excised, sliced into small pieces and used for protein identification.

SWATH-MS analysis

After denaturation, the proteins were alkylated with acrylamide and subjected to in-gel digestion with trypsin (0.01 mg mL⁻¹) by using the short GeLC approach.⁴⁶ The formed peptides were subjected to SPE using OMIX tips with a C18 stationary phase (Agilent Technologies) as recommended by the manufacturer and then resuspended in 30 μL of mobile phase containing iRT peptides (Biognosys AG) as internal standards. Five μL of each replicate sample was combined to obtain one pooled sample per experimental condition (in a total of four pools) to be used for protein identification and SWATH-MS (sequential windowed data independent acquisition of the total high-resolution mass spectra)-library generation.

Samples were analyzed on a Triple TOFTM 5600 System (ABSciex®) in two phases: information-dependent acquisition (IDA) of the pooled samples for protein identification, and SWATH acquisition of each individual sample for protein quantification (see details in the ESI†). A specific library of precursor masses and fragment ions was created by combining all files from the IDA experiments and used for subsequent SWATH processing. Libraries were obtained using Protein Pilot software (v5.1, ABSciex®) searching against a database composed of the genus Pseudomonas from the SwissProt database (released at June 2015).

SWATH data processing was performed using the SWATH™ processing plug-in for PeakView™ (v2.0.01, ABSciex®). Briefly, peptides were selected automatically from the library and up to 15 peptides with up to 5 fragment ions were chosen per protein. Quantitation was attempted for all proteins in the library file that were identified below 5% local false discovery rate (FDR) from ProteinPilot software (version 1.1.15) searching against a database composed of the genus Pseudomonas from the SwissProt database (released at June 2015). The effective concentrations of Ag⁺ and AgNPs inducing 10% or 20% of decrease (EC₁₀ and EC₂₀ with the respective 95% C.I.) in bacterial growth were calculated using PriProbit 1.63.⁴⁸ Data in percentages were arcsine root transformed to achieve normal distribution and homoscedasticity before analyses of variance.⁴⁹

The effects of cysteine and the form of silver (Ag⁺ or AgNPs) were tested by two-way analyses of variance (ANOVARs) followed by Dunnett’s post hoc tests to identify treatments that differed significantly from the control. Two-way ANOVARs were also used to test for the effects of AgNPs or Ag⁺ concentrations on antioxidant enzymes. One-way ANOVARs were used to test for the effects of each form of silver (Ag⁺ or AgNPs) on the protein contents. Analyses were done using Prism 7.0 for Windows (GraphPad Software Inc., CA, USA).

Fold changes of statistically significant proteins (one-way ANOVA, P < 0.05) were determined by log2 transformation of the ratio of protein levels under exposure to Ag⁺ or AgNPs versus control conditions.

Heatmap and clustering analyses of significantly altered proteins were performed using GProX (version 1.1.15). The proteins displayed in the heatmap were clustered according to their behavioural profiles. Clustering was performed using the unsupervised clustering fuzzy c-means algorithm implemented in the Mfuzz package,⁵¹ a soft clustering algorithm, noise-robust and well-fitted to the protein profile data. Gene Ontology (GO) enrichment analysis was performed and the most representative biological processes associated with each cluster were highlighted. GO annotations for the 59 statistically altered proteins were performed using Blast2GO (version 4.1.9) where protein sequences were loaded as a Sequences (FASTA) file. The BLAST was performed against the NCBI non-redundant (nr) protein database using the BLASTP method. GO annotations were exported for the Biological Process category and imported to the GProX software to perform the enrichment analysis. Each cluster was tested for overrepresented GO compared with cluster 3 (background) using the binomial statistical test with the Benjamini–Hochberg adjustment (P < 0.05). Significant effects were determined by PERMANOVA.

Principal component analysis (PCA) was applied to coordinate the alterations in the proteins and the activities of antioxidant enzymes (SOD, CAT, GST, and Gpx) according to the treatments (control, Ag⁺ and AgNPs). The proteins related to the stress response used in PCA were catalase-peroxidase (KatG) and alkyl hydroperoxide reductase subunit C (AhpC). Significant effects were determined by PERMANOVA. PCA was performed in PAST 3.14 for Windows (copyright Hammer & Harper, Ohio, USA). Results

Characterization of AgNPs and quantification of dissolved Ag⁺

The AgNPs in mineral medium showed a peak at 419 nm by UV-vis spectrophotometry. At the beginning of the experiment, AgNPs at the highest concentration showed two peaks
of hydrodynamic diameter (HDD) of smaller (average HDD <45 nm; area intensity of 8.3%) and larger size range (average HDD <310 nm; area intensity of 91.7%; Table 1). The peak with the larger hydrodynamic size range was probably the consequence of AgNP agglomeration due to interactions with the medium components. After 90 min of exposure to AgNPs, only one peak was observed and the size of the larger particles increased indicating increased NP agglomeration. The zeta potential of the AgNPs (−20.9 mV), the conductivity and the electrophoretic mobility did not change after 90 min of exposure (initial vs. final conductivity: 7.38 ± 0.09 vs. 7.41 ± 0.03 mS cm⁻¹; and mobility: −1.39 ± 0.05 vs. −1.39 ± 0.04 μm cm V⁻¹s⁻¹).

Changes in total silver quantification by ICP-MS in mineral medium either at the initial time (T0) or at the end of the experiment (T1 = 90 min) were not apparent (18.90 ± 0.71 μg L⁻¹ at T0 and 21.68 ± 3.03 μg L⁻¹ at T1; Table 1). Dissolved Ag⁺ quantification by ICP-MS revealed that, at the beginning of the exposure (T0), the concentration of Ag⁺ originating from the dissolution of AgNPs to the mineral medium (300 μg L⁻¹ in 50 mL) was 0.30 ± 0.07 μg L⁻¹; however, at the end of the experiment (T1), the concentration of dissolved Ag⁺ was lower than that at T0 (<0.2 μg L⁻¹; the detection limit was 0.06 μg L⁻¹).

Effects of Ag⁺ and AgNPs on the growth of Pseudomonas sp. M1

The specific growth rate of Pseudomonas sp. M1 was 0.80 h⁻¹; the growth was inhibited by the exposure to Ag⁺ and AgNPs. The increase in the concentration of Ag⁺ or AgNPs resulted in a dose-dependent reduction of the specific growth rate (Fig. 1). Inhibitory effects of Ag⁺ on the bacterial growth were more pronounced than those of AgNPs because EC₁₀ and EC₂₀ of Ag⁺ were ~4.4 and ~2.9 times lower than that of AgNPs, respectively (Table 2).

The role of Ag⁺ in the AgNP toxicity was further analysed by testing the effects of both forms of silver in the presence of an Ag⁺ ligand (cysteine). Exposure to cysteine alone did not affect the bacterial growth (P > 0.05; Fig. S1†). In the absence of cysteine, exposure to Ag⁺ or AgNPs at EC₂₀ of Ag⁺ were ~4.4 and ~2.9 times lower than that of AgNPs, respectively (Table 2).

The presence of cysteine did not alter the effect of AgNPs on Pseudomonas sp. M1 (P > 0.05) but alleviated the negative effect of Ag⁺, showing no difference in bacterial growth from control (P > 0.05; Fig. S1†).

Table 1  Zeta potential and hydrodynamic diameter (HDD) of AgNPs and total and dissolved Ag concentration in mineral medium with 0.4% lactate before (T0) and after 90 min (T1) of exposure to AgNPs at concentrations inhibiting 20% (EC₂₀) of the growth of Pseudomonas sp. M1. Mean ± SD, n = 3

<table>
<thead>
<tr>
<th>Time</th>
<th>Zeta potential (mV)</th>
<th>PdI</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Total Ag (μg L⁻¹)</th>
<th>Dissolved Ag (μg L⁻¹)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>HDD (nm)</td>
<td>Area intensity (%)</td>
<td>HDD (nm)</td>
<td>Area intensity (%)</td>
</tr>
<tr>
<td>T0</td>
<td>−20.9 ± 0.7</td>
<td>0.56 ± 0.12</td>
<td>307.4 ± 36</td>
<td>91.7 ± 2</td>
<td>42.2 ± 27</td>
<td>8.3 ± 2</td>
</tr>
<tr>
<td>T1</td>
<td>−20.9 ± 0.6</td>
<td>0.50 ± 0.31</td>
<td>657.8 ± 33</td>
<td>100 ± 0</td>
<td>—</td>
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</tbody>
</table>

PdI: polydispersity index.

Fig. 1 Effects of AgNPs (circles) or Ag⁺ (triangles) on the specific growth rate of Pseudomonas sp. M1. Data are percentages of the specific growth rate of the control culture in mineral medium with lactate at 30 °C. Mean ± SEM, n = 3.

Effects of Ag⁺ and AgNPs on the activity of antioxidant enzymes

After 90 min of exposure of Pseudomonas sp. M1 to Ag⁺ or AgNPs, the activities of all tested antioxidant enzymes, except for GST, increased significantly with concentration (two-way ANOVA, P < 0.05) of both silver forms (Fig. 2 and Table S1†). SOD and GPx activities increased significantly (P < 0.05) under Ag⁺ or AgNPs exposure, particularly at the highest concentrations (Fig. 2A and B). GST activity did not differ significantly from control (Fig. 2A and B). At the same concentration (50 or 100 μg L⁻¹), stimulation of SOD and GPx activities by Ag⁺ was stronger than by AgNPs (Fig. 2). When the enzyme activities were compared at the exposure concentrations closer to EC₁₀ (50 μg L⁻¹ for Ag⁺; 200 μg L⁻¹ for AgNPs), only SOD and GPx activities were significantly higher than in control upon exposure to Ag⁺ (303.6% and 295.2%, respectively; Fig. 2A), whereas AgNPs strongly induced the activities of GPx (310.8%), SOD (283.2%) and CAT (268.7%) (Fig. 2B). When the cells were exposed to 100 μg L⁻¹ Ag⁺ (near EC₂₀ of Ag⁺: 107.1 μg L⁻¹), maximum activity was observed for SOD (667.1%) followed by GPx (418.3%) and CAT (274.3%) (Fig. 2A). At 300 μg L⁻¹ AgNPs (EC₂₀ of AgNPs: 307.2 μg L⁻¹), the activity of SOD was highest (583.4%) followed by that of GPx (433.8%), whereas CAT activity decreased (135.5%) to the control level (Fig. 2B).

Effects of Ag⁺ and AgNPs on the proteome of Pseudomonas sp. M1

After 90 min of exposure of Pseudomonas sp. M1 to both forms of silver at concentrations (100 μg L⁻¹ Ag⁺ and 300 μg
Table 2 Concentrations of Ag⁺ and AgNPs inhibiting 10% (EC₁₀) and 20% (EC₂₀) of the growth of Pseudomonas sp. M1 grown in mineral medium with 0.4% lactate at 30 °C for 90 min. (95% C.I. in parenthesis)

<table>
<thead>
<tr>
<th>Stressor</th>
<th>EC₁₀ (μg L⁻¹)</th>
<th>EC₂₀ (μg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag⁺</td>
<td>51.2 (5.3–75.3)</td>
<td>107.1 (36.9–138.1)</td>
</tr>
<tr>
<td>AgNPs</td>
<td>225.7 (105.9–312.3)</td>
<td>307.2 (179.6–395.3)</td>
</tr>
</tbody>
</table>

L⁻¹ AgNPs) similar to their respective EC₂₀ on bacterial growth (EC₂₀ = 107.1 μg L⁻¹ for Ag⁺ and EC₂₀ = 307.2 μg L⁻¹ for AgNPs), a total of 166 proteins were identified through SWATH-MS (Table S2†). Out of these, the contents of 59 proteins (35.5%) were significantly altered after exposure to Ag⁺ or AgNPs (one-way ANOVA, \(P < 0.05\); Table S2†). Among these 59 proteins, 27 increased and 32 decreased after exposure to Ag⁺, whereas the opposite was found under AgNP exposure (i.e., 27 decreased and 32 increased) (Fig. S2†). Moreover, the majority (74.6%) of the proteins whose contents were significantly altered showed a similar pattern (22 proteins increased; 22 proteins decreased) under exposure to both Ag forms. The average fold change of positively altered proteins was higher under exposure to Ag⁺ (0.89-fold for Ag⁺ vs. 0.58-fold for AgNPs), whereas the opposite was found for negatively altered proteins (–0.71-fold for Ag⁺ vs. –1.87-fold for AgNPs).

A heatmap analysis shows the dynamic profiles of the proteins significantly altered (in the control and under exposure to Ag⁺ and to AgNPs; Fig. 3A). Proteins were clustered according to their behavioural profiles and 4 clusters were generated (Fig. 3B).

Cluster 1 was represented by 13 proteins, which increased under exposure to AgNPs (average fold change: 0.61) and decreased under exposure to Ag⁺ (average fold change: –0.29), except for 3 proteins (Table S2†). The contents of all proteins in cluster 2 (15 proteins) decreased under AgNP exposure (average: –1.44-fold), while under Ag⁺ exposure, 5 proteins increased (average fold change: 0.38) and 10 proteins decreased (average: –0.27). All proteins in cluster 3 (12 proteins) decreased significantly under Ag exposure (average: –2.39-fold by AgNPs and –1.43-fold by Ag⁺). Both silver forms stimulated (average: 0.56-fold by AgNPs and 1.05-fold by Ag⁺) the proteins from cluster 4 (19 proteins).

Relationships between oxidative stress enzymes and related proteins
PCA of overall responses of antioxidant enzymes and proteins involved in antioxidant activities in Pseudomonas sp. M1 under exposure to EC₂₀ of Ag⁺ or AgNPs showed that PC1 and PC2 explained 70.8% and 19.6% of the total variance, respectively (Fig. 4). Exposure of the cells to Ag⁺ and AgNPs led to significant effects on the activities of antioxidant enzymes and the contents of proteins with antioxidant activities (PERMANOVA, \(P < 0.05\)). PCA showed a clear discrimination between controls and treatments with Ag⁺ or AgNPs along the PC1 based on the responses of oxidative stress biomarkers. PC2 segregated Ag⁺ from AgNPs. With the exception of AhpC, all oxidative stress biomarkers were positively associated with Ag⁺ or AgNPs. CAT and KatG were closely associated with each other and were positively correlated to Ag⁺, whereas GPx, SOD and GST were associated with both treatments.

Discussion
Our study showed that short-term exposure to both Ag⁺ and AgNPs had negative impacts on the growth of Pseudomonas sp. M1, and the effects of Ag⁺ were more pronounced than those of AgNPs. This different sensitivity of the bacterium to Ag⁺ or AgNPs is in agreement with previous findings in aquatic microbes and other species of Pseudomonas.22,53 Short- and long-term exposure (30 min–24 h) to AgNPs led to toxic effects on aquatic Gram-positive and Gram-negative bacteria, although effective concentrations varied widely (45 ng–100 mg).15 The physicochemical properties of ENPs as well as the exposure time and species sensitivity can contribute to explain such differences.15 The responses of Pseudomonas sp. M1 to each silver form may be different from other strains or species due to putative adaptation to stressors existing in its natural habitat (Rhine River), which has been reported to be occasionally contaminated with metals and organic micropollutants.54–56 However, adaptive mechanisms of the
Fig. 3 Heatmap and cluster analysis of changes in proteins in *Pseudomonas* sp. M1 and GO enrichment analysis. The heatmap (A) shows the dynamic profiles of the 59 statistically significant proteins across the different experimental conditions (control, Ct; Ag⁺ or AgNPs, NP). The unsupervised cluster analysis (B) was performed considering standardization of the 59 altered proteins. An upper and lower ratio limit of log₂ (2) and log₂ (0.5) was used for inclusion into a cluster, and the 59 proteins were partitioned into 4 groups where the membership value represents how well the protein profile fits the average cluster profile. (C) Overrepresented biological processes of each cluster. Each cluster from (B) was tested for overrepresented GO compared with cluster 3 using a binomial statistical test with Benjamini-Hochberg adjustment (P < 0.05).
strain might be specific to the stressors existing in the environment, while the bacterial sensitivity to other stressors might not have been altered. The isolation site of *Pseudomonas* sp. M1 was not contaminated by silver. AgNPs (1–10 nm) can exhibit toxicity to *P. aeruginosa* by i) impairing membrane permeability and respiration, ii) interacting with sulphur- and phosphorus-containing macromolecules, leading to cellular damage, and iii) releasing Ag⁺ leading to additional bactericidal effects. In our study, it is conceivable that the actual toxic effects of AgNPs or Ag⁺ on *Pseudomonas* sp. might have been underestimated in the culture medium. The increased AgNP agglomeration might be the consequence of interactions with the medium components. Therefore, possible interactions between Cl⁻ and Ag⁺ released from AgNPs or AgNO₃ cannot be discarded. However, we want to point out that the dissolution of Ag⁺ was considerably low at the beginning of our experiment (~0.1%) and decreased further probably due to Ag⁺ biosorption to cells. This suggests a low contribution of Ag⁺ to the AgNP-induced stress. Moreover, the outcome of the complementary test with cysteine (Fig. S1†), a strong Ag⁺ ligand, further supported the theory that AgNPs induced direct toxicity to *Pseudomonas* sp. M1.

Common mechanisms of AgNPs in bactericidal activities involve i) cell wall disruption, ii) interaction with thiol-containing enzymes or peptides, iii) interaction with 30S ribosome and inhibition of protein synthesis, and iv) inhibition of DNA synthesis and induction of apoptosis. Our study showed that the short-term exposure of *Pseudomonas* sp. M1 to Ag⁺ or AgNPs stimulated the activities of antioxidant enzymes involved in the ascorbate–glutathione cycle, suggesting an attempt of cells to cope with the oxidative stress. The strong response of SOD suggested its crucial role in early defense against ROS, protecting the cells from Ag⁺- or AgNP-induced superoxide anion radical (O₂⁻•) by catalyzing its dismutation into H₂O₂. Chronic exposure to AgNPs (0.1–0.25 mg L⁻¹) increased the mRNA of SOD gene in the gill tissue of freshwater fish. However, the exposure to higher concentrations of AgNPs (1–1.5 mg L⁻¹) inhibited SOD activities in *P. aeruginosa* and *P. chlororaphis*, disrupting the antioxidant defense. In our study, the induced activities of GPx and CAT by exposure to both Ag forms suggested an efficient cellular detoxification mechanism by scavenging peroxides/hydroperoxides. GPx activity induced by CuONPs was higher in fungi isolated from metal-polluted streams compared to fungi from non-polluted streams, explaining the increase in their tolerance to CuONPs. Higher GPx activity may lead to the scarcity of GSH (reduced glutathione) by its conversion to GSSG (oxidized form), affecting the GSH pool required for cell protection against oxidative stress. Indeed, a considerable decrease in GSH (up to 80%) with exposure to AgNPs (1 mg L⁻¹) induced cytotoxicity in bacteria. In our study, CAT activity increased at the EC₂₀ of Ag⁺ and the EC₁₀ of AgNPs. A decrease in CAT activity was observed in *P. aeruginosa* upon longer exposure (12 h) to higher AgNP concentrations (1 mg L⁻¹), but not under short exposure (1 h) of *P. chlororaphis* to AgNPs (1.5 mg L⁻¹). In our study, GST activity was not triggered by either form of Ag, probably due to the efficient functioning of other antioxidant enzymes in cellular detoxification. The observed positive association between GPx and SOD activities under AgNP exposure suggested the contribution of these enzymes to coping with ROS. The pronounced activities of antioxidant enzymes under Ag⁺ exposure were consistent with the higher toxicity of the ionic form.

Our study provided evidence that Ag⁺ and AgNPs affected cellular targets and biological processes in *Pseudomonas* sp. M1. Out of 59 proteins, the contents of which were significantly altered after exposure to either Ag⁺ or AgNPs, 15 proteins showed different patterns against each Ag form. The proteomic profiles through GO enrichment analysis based on protein clustering further allowed the exploration of biological processes in *Pseudomonas* sp. M1 and provided the mechanistic insight into the stress induced by Ag⁺ or AgNPs (Fig. 3C). The most overrepresented process (12 proteins) under exposure to Ag⁺ or AgNPs was the translation, in which 8 proteins increased after exposure to AgNPs but decreased under Ag⁺ exposure (cluster 1, Fig. 3C). These proteins were mainly structural constituents of ribosomes which mediate the assembly of small or large ribosomal subunits (e.g. 30S ribosomal protein S8, RpsH; see Table S2†) and/or tRNA binding (e.g. 30S ribosomal protein S10, Rpsj; see Table S2†). These findings indicated that ionic and nanoparticulate forms of Ag interacted differently with this particular group of proteins. The remaining 4 proteins (clusters 2 and 3) were elongation factors (EFs; e.g. Tsf and Tuf, see Table S2†), which decreased under AgNP exposure more than under Ag⁺.
exposure. The decrease in the content of many elongation factors, including Efp, Tsf and Tuf1 (e.g. in Pseudomonas sp. M1 under exposure to phenol⁴⁰ in P. stutzeri exposed to FeNPs⁶¹), may indicate a compromised protein synthesis due to the stress caused by the nanoparticulate form of Ag. A strong functional association between proteins was obtained in P. aeruginosa using STRING database,⁶² selecting the networks of the proteins altered by Ag⁺ or AgNPs in Pseudomonas sp. M1 (Fig. S3†). Moreover, if the polypeptide chain elongation process was affected by AgNPs, defective proteins would be produced and, hence, the performance of the cellular machinery responsible for maintaining protein homeostasis may be altered. In fact, we found an increase in the content of ribosomal constituents under AgNPs exposure which could represent the attempt of cells to counteract incorrect protein formation. Although the process of amino acid synthesis was not featured by GO enrichment analysis, the content of related proteins (e.g. LeuC, LeuD and LeuB; cluster 3) decreased under exposure to AgNPs more than to Ag⁺, affecting the synthesis of proteins and leucine-based enzyme activities. Indeed, a very strong functional association between LeuD and LeuB was obtained in P. aeruginosa using the STRING database when the interaction networks among the proteins altered by Ag⁺ or AgNPs in Pseudomonas sp. M1 were selected (Fig. S3†). A decrease in the content of proteins involved in amino acid metabolism by Ag⁺ and graphene-based AgNPs was reported in P. aeruginosa.⁶³ GO enrichment analysis further supported these findings, demonstrating the importance of other proteins in Pseudomonas sp. M1, particularly those associated with the process of protein folding (cluster 4, Fig. 3C) under Ag⁺ exposure. This process is accomplished by chaperones, a group of ubiquitous stress responsive proteins that are mainly responsible for proteostasis. Among the functions ensured by these proteins are the prevention of misfolding and promotion of refolding and proper assembly of unfolded polypeptides generated under stress conditions (e.g. GroEL, see Table S2†) or the binding to nascent proteins, assisting in their correct folding (e.g. DnaK, see Table S2†). STRING analysis of P. aeruginosa based on the proteins altered by Ag⁺ in Pseudomonas sp. M1 showed strong interactions between GroEL and DnaK (Fig. S3†). An increase in GroEL content was also reported in P. stutzeri after exposure to FeNPs.⁶¹ DnaK can play a protective role against many stressors, including AgNPs, diminishing the antimicrobial effects and increasing the tolerance of P. aeruginosa.⁶⁴ Cells of Pseudomonas sp. M1 present low levels of these chaperones, but their levels increase under stress, contributing to their tolerance and survival in stressful conditions.⁴⁰ Thus, in our study, the increased content of these proteins suggests their involvement in preventing protein misfolding under the stress induced by Ag in Pseudomonas sp. M1.

Two of the differentially altered proteins by either forms of Ag had antioxidant roles (KatG and AhpC, see Table S2†) helping to maintain cell-redox homeostasis. Moreover, the activities of CAT and the alterations in the protein content of KatG (1.58-fold for Ag⁺ vs. 0.74-fold for AgNPs) were closely associated with each other and positively correlated to Ag⁺. This suggests that more hydroperoxides were induced by exposure to Ag⁺ and that CAT played a crucial role in the cellular detoxification in Pseudomonas sp. M1. Increased expression level of catalase (KatA, 1.69-fold) after exposure (24 h) to AgNPs (at 1.2 mg L⁻¹) was also reported in P. aeruginosa.³⁷ AhpC is a key component of a large family of thiol-specific antioxidant proteins known to scavenge reactive oxygen, nitrogen and sulphur species and organic hydroperoxides by reducing to diol forms. An increase in the content of AhpC can explain the tolerance of organisms to multiple stressors,⁴⁰,⁶⁵ as observed in Pseudomonas sp. after 24 h of exposure to 100 μg L⁻¹ AgNPs.⁶⁶ In our study, the content of AhpC decreased under the exposure to both forms of Ag, mainly under AgNP exposure (~0.38-fold for Ag⁺; ~2.68-fold for AgNPs), probably due to shorter exposure to a higher concentration of nanoparticles. Cosgrove et al.⁶⁷ reported greater catalase expression provoked by a mutation in AhpC, suggesting its compensatory role in oxidative stress. Hence, it is possible that the decrease in the AhpC that had occurred in Pseudomonas to privilege other antioxidant enzymes against Ag-induced oxidative stress.

In Pseudomonas sp. M1, the content of most proteins associated with the tricarboxylic acid cycle (clusters 2 and 4, Fig. 3C) increased under Ag⁺ exposure, but decreased under AgNP exposure (e.g. aconitate hydratase B – AcmB and isocitrate dehydrogenase [NADP] – Icd), suggesting that different pathways of carbohydrate and energy metabolism were affected by Ag⁺ and AgNPs. STRING analysis also showed a strong interaction between AcmB and Icd (Fig. S3†). The exposure to graphene-based AgNPs altered the content of proteins involved in carbohydrate and energy metabolism in P. aeruginosa.⁵³ A decrease in AcmB by other metal NPs was also reported in P. stutzeri.⁶¹ In contrast, the increase in the content of those proteins under Ag⁺ exposure in Pseudomonas sp. M1 suggested an adaptive mechanism in energy consumption and carbohydrate metabolism under Ag stress.

Transmembrane transport was another overrepresented process displayed by GO analysis in Pseudomonas sp. M1 (cluster 4) in which proteins (e.g. membrane protein, porins and outer membrane protein W, see Table S2†) increased under Ag⁺ exposure more strongly than under AgNP exposure. This indicates that these proteins were mainly targeted by Ag⁺, affecting the membrane transport and facilitating Ag⁺ entrance into bacterial cells.

The processes of pathogenesis and of negative regulation of single-species biofilm formation were also revealed by GO enrichment analysis (cluster 4, Fig. 3C). Although no designation related to this process (as a biological process annotated in UniProt) has been attributed to the proteins belonging to cluster 4, there is evidence that virulence factors related to pathogenesis can also be encoded in the genomes of non-pathogenic bacteria.⁶⁸ Moreover, Pitondo-Silva et al.⁶⁹ demonstrated a co-occurrence of resistance to antibiotics and heavy metals associated with the presence of virulence genes in P. aeruginosa, although the influence of other genes and/
or mechanisms related to metal resistance could not be discarded. The process of biofilm formation, whose role in protecting bacteria from antibiotics is well known, was negatively regulated in Pseudomonas sp. M1 in our study. Singh et al. demonstrated that AgNPs affect the P. aeruginosa biofilm by disabling quorum sensing, a communication process used by the strain to regulate virulence and biofilm formation. Because biofilm formation requires a considerable amount of energy in producing extracellular polymeric substances, cells of Pseudomonas sp. M1 may have prioritized mechanisms/processes (e.g. pathogenesis/virulence) to save energy to deal with the stress induced by both silver forms.

Conclusions

Our study showed stronger impacts of the ionic form than the nanoparticulate form of Ag on the growth of Pseudomonas sp. M1. However, the low extracellular dissolution of Ag+ from AgNPs supported the low contribution of dissolved Ag+ to the stress induced by AgNPs. Our results also demonstrated the involvement of antioxidant enzymes of the ascorbate–glutathione cycle in coping with the oxidative stress induced by both forms of Ag. Proteomic analysis allowed us to identify several proteins, including chaperones, transmembrane transporters, and proteins related to the metabolism of proteins, amino acids, carbohydrates and energy, pointing to them as potential biomarkers of the stress induced by Ag+ and/or AgNPs. The process of translation was most representative of AgNP exposure, whereas protein folding together with transmembrane transport was the most relevant under Ag+ exposure. Moreover, it was possible to conclude that each form of Ag induced different adaptive responses in the metabolic, energetic and stress pathways in Pseudomonas sp. M1. Overall, our study supports the value of proteomics for elucidating the mechanisms of responses to ENPs and for further assessing the ecotoxicological risk of nanomaterials.

Conflicts of interest

The authors declare no conflict of interests related to this study. They alone are responsible for the contents and writing of the paper. There are no conflicts to declare.

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